LEXOGEN

Enabling complete transcriptome sequencing

SENSE™

Making sense of RNA sequencing

mRNA-Seq Library Prep Kit

User Guide

Catalog Numbers:
001.08 (mRNA-Seq Library Prep Kit for Illumina, 8 rxn)
001.24 (mRNA-Seq Library Prep Kit for Illumina, 24 rxn)
002.08A (In-line Barcode Kit for Illumina, 8 rxn/barcode, 12 barcodes)
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003.08A-H (External Barcode Kit for Illumina, 8 rxn/barcode, 12 barcodes; sets A-H)
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1. Overview

This SENSE mRNA-Seq kit is an all-in-one library preparation protocol designed to generate Illumina-compatible libraries from total RNA within 4 hours. The SENSE protocol maintains strand-specificity (>99.9%) and allows the mapping of reads to their corresponding strand on the genome, enabling the discovery and quantification of antisense transcripts and overlapping genes. SENSE includes an integrated poly(A) selection, so prior rRNA depletion is not required. Insert size can be varied during the library preparation protocol itself, meaning that size selection with additional kits is not necessary. Optional multiplexing of libraries can be carried out using up to 12 in-line barcodes or up to 96 external barcodes. Libraries are compatible with both single-end and paired-end sequencing reagents.

The SENSE protocol consists of a highly specific bead-based poly(A) selection step which removes almost all traces of rRNA, tRNA, and non-polyadenylated RNA. Information regarding input RNA requirements can be found in Appendix A (p.18).

Library production is initiated by the random hybridization of starter/stopper heterodimers to the poly(A) RNA still bound to the magnetic beads. These starter/stopper heterodimers contain Illumina-compatible linker sequences. A single-tube reverse transcription and ligation reaction extends the starter to the next hybridized heterodimer, where the newly-synthesized cDNA insert is ligated to the stopper. As the insert size is determined by the distance between starter/stopper binding sites RNA fragmentation is not required. Therefore spurious second strand synthesis from the 5' ends of fragments is absent providing the basis for the excellent strand-specificity of the SENSE protocol.

Second strand synthesis is performed to release the library from the beads, and the library is then amplified, introducing the sequences required for cluster generation (see Appendix E, p.28, for a schematic representation of the finished library). Library quantification can be performed with standard protocols and is further discussed in Appendix C (p.22). Libraries are compatible with single-end or paired-end sequencing. Barcodes can be introduced either as in-line barcodes at the beginning of each read or as standard external barcodes (Appendix D, p.24). External barcodes require a separate sequencing reaction whereas in-line barcodes do not. Data can be analyzed with a number of standard bioinformatic pipelines. Special considerations for the analysis of SENSE data, such as read orientation, are presented in Appendix F (p.31).

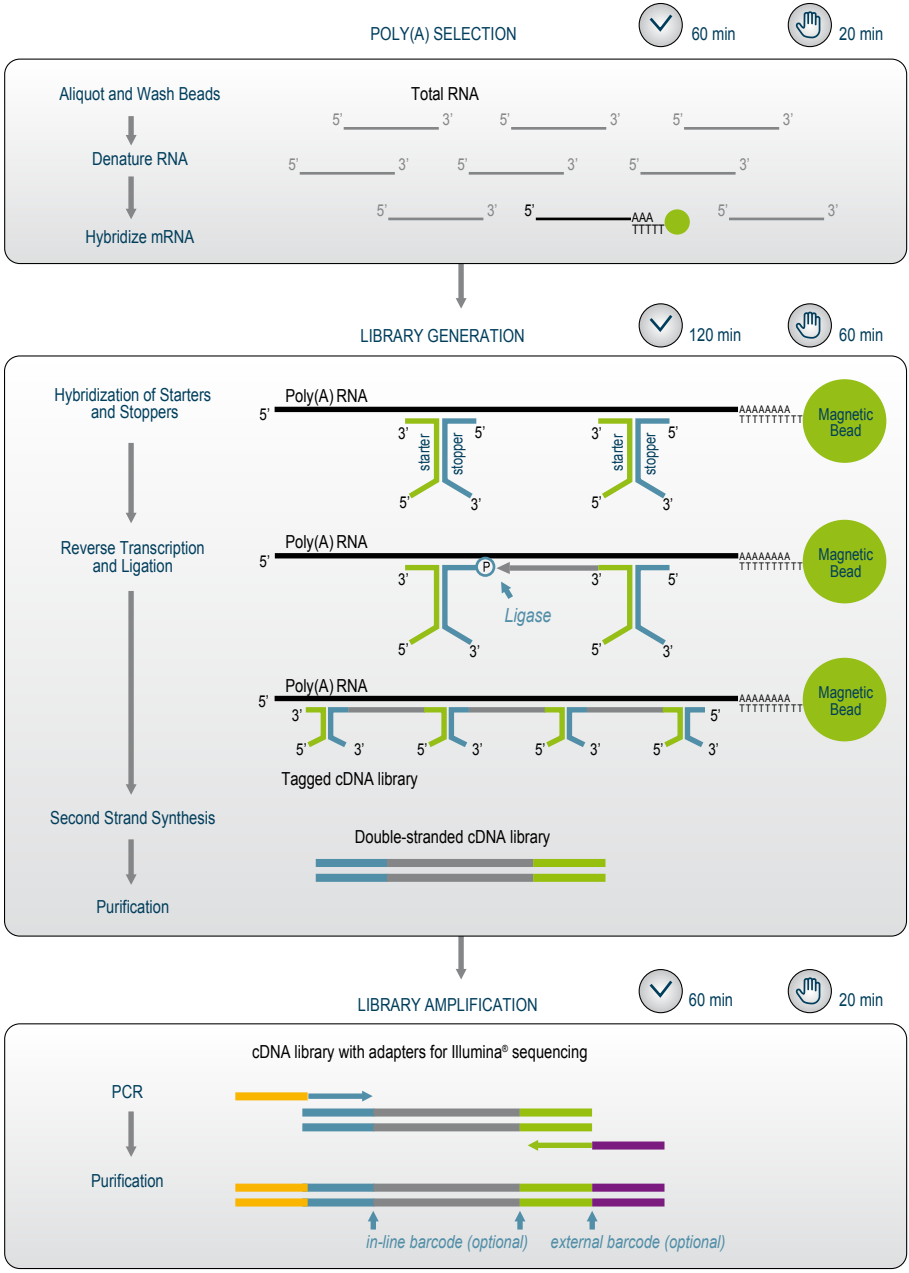


Figure 1. Schematic overview of the SENSE workflow.

2. Kit Components and Storage Conditions

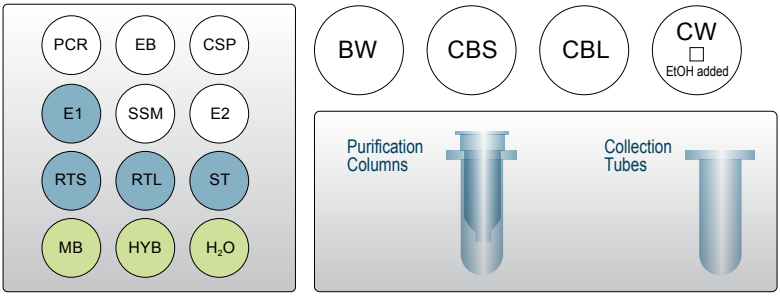


Figure 2. Location of kit contents.

Kit Components	Tube Label	Volume needed for		Storage
		8 preps	24 preps	
Magnosphere MS150/ oligodT Beads	**MB**	80 µl	240 µl	-20 °C
RNA Hybridization Buffer	**HYB**	80 µl	240 µl	-20 °C
Molecular Biology Grade Water	**H_2O**	160 µl	480 µl	-20 °C
Reverse Transcription and Ligation Mix Short	**RTS**	120 µl	360 µl	-20 °C
Reverse Transcription and Ligation Mix Long	**RTL**	120 µl	360 µl	-20 °C
Starter/Stopper Mix (No In-line Barcode)	**ST**	16 µl	48 µl	-20 °C
Enzyme Mix 1	**E1**	24 µl	72 µl	-20 °C
Second Strand Synthesis Mix	**SSM**	72 µl	216 µl	-20 °C
Enzyme Mix 2	**E2**	24 µl	72 µl	-20 °C
PCR Mix	**PCR**	64 µl	192 µl	-20 °C
Elution Buffer	**EB**	464 µl	1390 µl	-20 °C
Customized Sequencing Primer 100 µM	**CSP**	80 µl	240 µl	-20 °C
Bead Wash Buffer	**BW**	6.4 ml	19.2 ml	RT
Column Binding Buffer Short	**CBS**	3.84 ml	11.52 ml	RT
Column Binding Buffer Long	**CBL**	2.56 ml	7.68 ml	RT
Column Wash Buffer	**CW**	6.4 ml*	19.2 ml*	RT

*including ethanol (to be added by user)

Upon receiving the SENSE kit, remove the smaller inner box and store it in a -20 °C freezer. The rest of the kit components should be stored at room temperature and protected from light. Before use, check the contents of **BW**, **CBS**, **CBL**, and **CW**, which may precipitate during shipping. If a white precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

Cat. No. 001.08 (8 preps): Add 6 ml absolute ethanol to **CW** and shake to combine.
Cat. No. 001.24 (24 preps): Add 18 ml absolute ethanol to **CW** and shake to combine.

3. User-supplied Consumables and Equipment

Check to ensure that you have all of the necessary materials and equipment before beginning library preparation. All reagents, equipment and labware must be free of nucleases and nucleic acid contamination.

Reagents

- Absolute ethanol, add to column wash (**CW**) solution.

Equipment

- Magnetic rack.
- Benchtop centrifuge (12,000 x g, rotor compatible with 1.5 ml tubes).
- Calibrated single-channel pipettes for handling 1 µl to 1000 µl volumes.
- Thermomixer for 1.5 ml tubes (dry bath incubator with shaking function).
- Thermocycler.
- UV-spectrophotometer to quantify RNA.

Optional equipment

- Automated microfluidic electrophoresis station (Agilent Technologies 2100 Bioanalyzer).
- qPCR machine and library standards (for library quantification).
- Benchtop fluorometer and appropriate assays (for RNA quality control and library quantification).
- Agarose gels, dyes, and electrophoresis rig (for RNA quality control).

Labware

- Suitable pipette tips (pipette tips with aerosol barriers recommended).
- 1.5 ml reaction tubes, low binding, certified ribonuclease-free.
- 200 µl PCR tubes or 96 well plates and caps or sealing foil.
- Vortex mixer.
- Ice bath or ice box, ice pellets, benchtop cooler (-20 °C for enzymes).

The complete set of materials, reagents and labware necessary for RNA extraction and quality control is not listed. Consult Appendix A (p.18) for more information on RNA quality.

Consult Appendix C (p.22) for information on library quantification methods.

4. Guidelines

RNA Handling

- RNases are ubiquitous and special care should be taken throughout the procedure to avoid RNase contamination.
- Use commercial ribonuclease inhibitors (i.e. RNasin, Promega Corp.) to maintain RNA integrity when storing samples.
- Use a sterile and RNase-free workstation or laminar flow hood if available. Please note that RNases may still be present on sterile surfaces, and that autoclaving does not completely eliminate RNase contamination. Before starting a library preparation, clean your work space, pipettes, and other equipment with RNase removal spray (such as RNaseZap, Ambion Inc.) as per the manufacturer's instructions.
- Protect all reagents and your RNA samples from RNases on your skin by wearing a clean lab coat and fresh gloves. Change gloves after making contact with equipment or surfaces outside of the RNase-free zone.
- Avoid speaking above opened tubes. Keep reagents closed when not in use to avoid airborne RNase contamination.

Bead Handling

- Beads are stored at -20 °C and must be be resuspended after thawing. Beads can be resuspended by pipetting up and down several times or by vortexing. When properly resuspended, the solution should have a uniform brown color with no visible clumping on the walls or bottom of the tube.
- Beads may stick to certain pipette tips, in which case removing the beads from the inside of the tip may be impossible. Avoid resuspending by repeated pipetting and instead resuspend by vortexing if this occurs with your tips.
- Beads are superparamagnetic and are collected by placing the tube in a magnetic stand. The time required for complete separation will vary depending on the strength of your magnets, tube thickness, viscousity of the solution, and the proximity of the tube to the magnet. Separation time may need to be adjusted accordingly. When fully separated, the supernatant should be completely clear and the beads collected at one point or line on the wall of the tube.
- To remove the supernatant the tube containing the beads has to stay in close contact with the magnet. Do not remove the tube from the magnetic stand when removing the supernatant, as the absence of the magnet will cause the beads to go into solution again.
- In general, beads should not be centrifuged during the protocol. However, should liquid

condense (e.g. after step 16) or become entrapped in the cap or drops of fluid stay on the side of the reaction tube, centrifugation at 2,000 x g for 30 sec should be carried out before placing the tube on the magnetic rack.

- Allowing the beads to dry out can damage them. Always keep the beads in suspension except for the short period after withdrawing the supernatant but before adding the next reagent. Beads can be resuspended by vortexing but make sure that beads are not deposited on the tube walls above the level of the liquid, where they can dry during incubation. If necessary, stuck beads can be collected by centrifuging the tube briefly with a benchtop centrifuge.

General

- Unless explicitly mentioned, all steps should be carried out at a room temperature (RT) between 20 °C and 25 °C. Results may be negatively impacted if the protocol is performed at temperatures outside of this range. While reaction set-up is often performed at RT, incubation temperatures are explicitly defined and must be strictly adhered to.
- To further increase reproducibility, centrifugation should be performed at 18 °C. If a refrigerated centrifuge is not available, centrifugation can be carried out at RT.
- Ensure that adequate volumes of all reagents and the necessary equipment is available and set to the proper temperatures before beginning the protocol.
- Make sure to pre-heat thermomixers (dry bath incubators) well in advance.
- Perform all pipetting steps with calibrated pipettes and always use fresh tips. Pipette carefully to avoid foaming as some solutions contain detergents.
- Thaw all necessary buffers at room temperature or as indicated in the preparation tables at the beginning of each step of the detailed protocol. Mix reagents well by vortexing or pipetting repeatedly and centrifuge briefly with a benchtop centrifuge to collect contents before use.
- Keep enzyme mixes at -20 °C until right up before use or store in a -20 °C benchtop cooler.
- Steps requiring a thermocycler have been tested with a maximum ramp speed of 5 °C/sec before denaturation and extension and 2.5 °C/sec during primer annealing. While these ramp speeds are typical for most modern thermocyclers, some models can exceed these rates, and ramp speed may need to be decreased to ensure efficient annealing.

Pipetting and handling of (viscous) solutions

- Enzymes, RTS, and RTL are viscous solutions which require care to pipette accurately. Quickly spin down the tubes to collect all liquid at the bottom of the tube. Be sure to pipette slowly and check the graduation marks on your pipette tips when removing an aliquot.
- When drawing up liquid the tip should be dipped 3 to 5 mm below the surface of the liquid,

always at a 90 degree angle. Do not dip the tip in any further as viscous solutions tend to stick to the outside of the pipette tip.

- Any residual liquid adhering to the tip should be removed by sliding the tip up the wall or edge of the tube from which the liquid was taken. Spin down the tube afterwards again to ensure that all liquid is collected at the bottom of the tube for further storage.
- When dispensing, the pipette should be held at a 45 degree angle, and the tip placed against the side of the receiving vessel.
- When pipetting liquids from bottles take special care that only the sterile pipette tip touches the bottle opening to prevent introducing RNAses or other contaminants. Tips are sterile whereas the pipette itself is not. If necessary tilt the bottle to bring the liquid closer to the opening and facilitate pipetting.

5. Detailed Protocol

5.1 Poly(A) Selection

Preparation

Aliquot and Wash Beads	Denature RNA	Hybridize mRNA
MB – thawed at RT **HYB** – thawed at RT **BW** – stored at RT	**Total RNA** – thawed on ice **H_2O** – thawed at RT	**BW** – stored at RT
Magnetic rack	Thermocycler 60 °C 1 min 25 °C hold	Thermomixer set to 25 °C 1,250 rpm

Aliquot and Wash Beads

SENSE uses Magnosphere MS150/oligodT beads from JSR Life Sciences. The magnetic beads must be washed before use. All steps are performed at room temperature.

1. Mix the beads (**MB** ●) well. Transfer 10 µl of the resuspended beads per library preparation into a new 1.5 ml tube. Beads can be washed as a batch if multiple library preparations are required.
2. Place the tube in a magnetic rack and let the beads collect for 2 minutes. Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.
3. Remove the tube from the magnetic rack and add 200 µl bead wash buffer (**BW**) per library preparation. Resuspend the beads and transfer the tube to the magnetic rack. Let the beads collect for 1 minute, remove and discard the supernatant.
4. Repeat this washing step once (for a total of two washes).
5. Resuspend the beads in 10 µl RNA hybridization buffer (**HYB** ●) per library preparation. Pipette and mix carefully to avoid introducing air bubbles.

Denature RNA

RNA samples are briefly heated to resolve secondary structures and promote efficient hybridization. For information on appropriate amounts of total RNA input as well as RNA quantification and quality control see Appendix A (p.18).

6. Dilute 500 ng to 2 µg of total RNA to a volume of 10 µl with RNase-free water (**H_2O** ●).
7. Denature RNA samples using a thermocycler at 60 °C for 1 minute and then hold at 25 °C. Do not cool samples excessively or place denatured RNA on ice.

Hybridize mRNA

The denatured total RNA is incubated with the washed beads, which specifically bind polyadenylated RNAs. RNAs lacking a poly(A) tail are then washed away, leaving only purified poly(A) RNA hybridized to the beads.

8	Add the 10 µl of denatured RNA to 10 µl of washed beads and incubate using a thermomixer at 25 °C for 20 minutes with 1,250 rpm agitation.
9	Transfer the solution to a magnetic rack, and remove and discard the supernatant.
10	Remove the tube from the magnetic rack and add 100 µl bead wash buffer (**BW**). Resuspend the beads and mix well. Incubate using a thermomixer at 25 °C for 5 minutes with 1,250 rpm agitation. Collect the beads by placing the tube onto a magnetic stand for 2 minutes. Remove and discard the supernatant.
11	Repeat this washing step once (for a total of two washes).

5.2 Library Generation

Preparation

Reverse Transcription and Ligation	Second Strand Synthesis	Purification
RTS } thawed on thermomixer, **RTL** } 5 min, 25 °C, 1,250 rpm **ST** - thawed at RT **E1** - keep on ice or at -20 °C **BW** - stored at RT **H_2O** - thawed at RT	**SSM** - thawed at RT **E2** - keep on ice or at -20 °C	**CBS** - stored at RT **CBL** - stored at RT **CW** - stored at RT **EB** - thawed at RT
Thermomixer set to 25 °C 1,250 rpm Magnetic rack	Thermocycler 98 °C for 90 sec 65 °C for 60 sec 72 °C for 5 min 25 °C ∞	Benchtop centrifuge Column (1 per sample) Collection tubes (2 per sample)

Reverse Transcription and Ligation

The starter/stopper heterodimer mix is hybridized to the RNA, and reverse transcription and ligation is performed, generating short cDNA fragments with linker sequences at either end.

12. After removing the supernatant from the last wash, add 15 µl reverse transcription and ligation mix **RTS** ● or **RTL** ●. **ATTENTION: RTS** ● is used for sequencing runs up to 150 nt single-end or 50 nt paired-end; **RTL** ● is used for sequencing runs ≥150 nt single-end or ≥100 nt paired-end. Please also consult Appendix B: Adjusting Library Size (p.21).
13. Add 2 µl starter/stopper heterodimer (**ST** ●). For multiplexed libraries with in-line barcoding, replace **ST** ● with **ST1** through **ST12** ● as described in Appendix D (p.23). Mix by vortexing.
14. Incubate at 25 °C for 5 minutes using a thermomixer with 1,250 rpm agitation.
15. Add 3 µl of enzyme mix 1 (**E1** ●), mix by vortexing and incubate at 25 °C for an additional 2 minutes at 1,250 rpm.
16. Set the thermomixer to 37 °C and incubate for one hour with 1,250 rpm agitation.
17. Apply 100 µl bead wash buffer (**BW**) to the RT/ligation reaction and mix thoroughly. Collect the beads with a magnetic rack for 2 minutes, remove and discard the supernatant.
18. Apply 100 µl bead wash buffer (**BW**) to the beads and resuspend by pipetting or vortexing gently. Collect the beads with a magnetic rack, remove and discard the supernatant.
19. After removing the supernatant from the second wash, resuspend the beads in 10 µl RNase-free water (**H_2O** ●).

Second Strand Synthesis

During this step the library is converted to dsDNA and is freed from the hybridized RNA by both the hydrolysis of the RNA and the second strand synthesis reaction itself.

20. Transfer the resuspended beads to a PCR tube or plate containing 9 µl second strand synthesis mix (**SSM** ○).
21. Add 1 µl enzyme mix 2 (**E2** ○) and mix well.
22. Conduct one cycle of thermocycling with the following program: 98 °C for 90 seconds, 65 °C for 60 seconds, 72 °C for 5 minutes, hold at 25 °C.

Purification

The double-stranded library is column-purified to remove the magnetic beads and second strand synthesis reaction components.

ATTENTION: Two different column binding buffers **CBS** and **CBL** are provided to further refine library size during column purification. For appropriate mixing of **CBS** and **CBL** please consult Appendix B: Adjusting Library Size (p.21).

23. Add a total of 160 µl column binding buffer (x µl **CBS** and y µl **CBL**, see Appendix B, p.21) to the reaction, mix well, and transfer the solution to a purification column placed in a 2 ml collection tube. Centrifuge for 1 minute at 12,000 x g at 18 °C.
24. Transfer the purification column into a new 1.5 ml tube. Do not discard the collection tube.
25. Apply 20 µl elution buffer (**EB** ○) to the column. Incubate at room temperature for 1 minute and centrifuge for 2 minutes at 12,000 x g at 18 °C to elute the library.
26. Add a total of 160 µl column binding buffer (same mix as used in step 23: x µl **CBS** and y µl **CBL**, see Appendix B, p.21) to the eluted 20 µl, mix well, and reload the solution onto the same purification column. Place the purification column back into in the original collection tube. Centrifuge for 1 minute at 12,000 x g at 18 °C.
27. Apply 200 µl of column wash buffer (**CW**) to the column and centrifuge for 1 minute at 12,000 x g at 18 °C.
28. Repeat this washing step once (for a total of two washes).
29. Transfer the column to a fresh collection tube. Centrifuge for 2 minutes at 12,000 x g at 18 °C to dry the column.
30. Transfer the column to a new 1.5 ml tube and apply 13 µl elution buffer (EB ○) to the column. Incubate at room temperature for 1 minute and centrifuge for 2 minutes at 12,000

x g at 18 °C to elute the library. **ATTENTION:** If a qPCR is intended to determine the exact cycle number of the endpoint PCR, apply 23 µl elution buffer (EB ○) to the column. For further details please refer to Appendix A (p.18).

31 After elution, libraries can be stored at -20 °C for later amplification.

5.3 Library Amplification

Preparation

PCR	Purification
PCR – thawed at RT **E2** – keep on ice or at -20 °C	**CBS** – stored at RT **EB** – thawed at RT **CW** – stored at RT
Thermocycler 98 °C for 30 sec 98 °C for 10 sec 65 °C for 20 sec } 8 -10 x 72 °C for 30 sec } see Appendix B, p.21 72 °C for 2 min 10 °C ∞	Benchtop centrifuge Column (1 per sample) Collection tubes (2 per sample)

PCR

The library is amplified to add the complete adaptor sequences required for cluster generation and to generate sufficient material for quality control and sequencing.

32 Transfer 10 µl of the eluted library to a PCR tube or plate containing 8 µl PCR mix (PCR ○) or 8 µl of the respective external barcode mix (PCR01-96) if multiplexing of libraries is intended. (External barcode mixes (003.08 A-H and 003.24 A-H) are sold separately and contain all reagents necessary for the PCR with the exception of the enzyme mix.)

33 Add 2 µl of enzyme mix 2 (**E2** ○) and mix thoroughly.

34 Conduct 8 to 10 cycles of PCR with the following program: Initial denaturation at 98 °C for 30 seconds, 8 to 10 cycles of 98 °C for 10 seconds, 65 °C for 20 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 2 minutes, hold at 10 °C. **ATTENTION:** Cycle numbers vary depending on the **CBS/CBL** mixture used in steps 23 and 26. Please refer to the table in Appendix B (p.21).

Purification

The finished library is purified from PCR components that can interfere with quantification.

35	Add 160 µl of column binding buffer (**CBS**) to the reaction, mix well, and transfer the solution to a column placed in a 2 ml collection tube. Centrifuge for 1 minute at 12,000 x g at 18 °C.
36	Apply 200 µl of column wash buffer (**CW**) to the column and centrifuge for 1 minute.
37	Repeat this washing step once (for a total of two washes).
38	Remove the column and transfer to a fresh collection tube. Centrifuge for 2 minutes at 12,000 x g at 18 °C to dry the column.
39	Transfer the column to a new 1.5 ml tube and apply 15 µl elution buffer (**EB** O) to the column. Incubate at room temperature for 1 minute and centrifuge for 2 minutes at 12,000 x g at 18 °C to elute the library.
40	At this point, the libraries are finished and ready for quality control (Appendix C, p.22), pooling (for multiplexed SENSE libraries; see Appendix D, p.24), and cluster generation.

6. Short Procedure

60 min	Poly(A) Selection
☐☐ wash 10 µl beads 2 times with 200 µl **BW** ☐ resuspend beads with 10 µl **HYB**	Aliquot and Wash Beads
☐ dilute 500 ng to 2 µg total RNA in 10 µl with **H_2O** ☐ incubate for 1 min at 60 °C, hold at 25 °C	Denature RNA
☐ add RNA (10 µl) to beads (10 µl) ☐ incubate for 20 min at 25 °C / 1,250 rpm ☐☐ wash 2 x for 5 min at 25 °C / 1,250 rpm with 100 µl **BW** ☐ withdraw supernatant	Hybridize mRNA

120 min	Library Generation
☐ add 15 µl **RTS** or **RTL** (see p.21) and resuspend beads ☐ add 2 µl **ST** and incubate for 5 min at 25 °C / 1,250 rpm ☐ add 3 µl **E1** and incubate for 2 min at 25 °C / 1,250 rpm ☐ raise temp. to 37 °C and incubate for 1 h / 1,250 rpm ☐☐ wash twice with 100 µL **BW** ☐ resuspend beads with 10 µl **H_2O**	Reverse Transcription and Ligation
☐ add 9 µl **SSM** and 1 µl **E2** ☐ incubate: 98 °C / 90 sec, 65 °C / 60 sec, 72 °C / 5 min	2nd Strand Synthesis
☐ add .... µl **CBS** and .... µl **CBL** (see p.21), apply to column, centrifuge 1 min ☐ exchange collection tube with 1.5 ml tube ☐ add 20 µl **EB** to column, incubate 1 min at RT, centrifuge 2 min ☐ add .... µl **CBS** and .... µl **CBL** to eluate, reload onto same column, transfer column into collection tube, centrifuge 1 min ☐☐ add 200 µl **CW**, centrifuge 1 min, repeat once ☐ transfer column into a fresh collection tube, centrifuge 2 min ☐ exchange collection tube with 1.5 ml tube ☐ add 13 µl **EB** to column, incubate 1 min at RT, centrifuge 2 min	Purification

60 min	Library Amplification
☐ add 8 µl **PCR** and 2 µl **E2**, mix ☐ PCR: 98 °C for 30 sec 98 °C for 10 sec 65 °C for 20 sec 72 °C for 30 sec } 8 -10 x (see p.21) 72 °C for 2 min; 10 °C hold	PCR
☐ add 160 µl **CBS**, centrifuge 1 min ☐☐ add 200 µl **CW**, centrifuge 1 min, repeat once ☐ exchange collection tube, centrifuge 2 min ☐ exchange collection tube with 1.5 ml tube ☐ add 15 µl **EB** to column, incubate 1 min at RT, centrifuge 2 min	Purification

7. Appendix A: RNA Requirements - PCR Cycles

RNA amount

High quality mRNA-Seq data relies on high quality input RNA. The amount of total RNA required for SENSE depends on the poly(A) RNA content of the sample in question. This protocol was tested extensively with various mouse tissues and human reference RNA. Typical inputs of 500 ng total RNA for mRNA-rich tissues (such as kidney, liver, and brain) or 2 µg total RNA for tissues with lower mRNA content (such as lung and heart) generate high quality libraries for single-end 50 nt sequencing (SR50) with 8 cycles of library amplification. For other library sizes PCR cycles need to be adjusted as described in the table of Appendix B (p.21).

The input requirements for your particular experiment may be different, and we have included extra reagents for library amplification and purification to assist with optimization. If RNA input is not sufficient, either due to naturally low poly(A) RNA content or degraded RNA, additional cycles of library amplification may be necessary. However, as additional cycles of library amplification may increase the proportion of PCR duplicates, it is more desirable to increase the amount of input RNA (if possible for your application) rather than to rely on extra PCR cycles to increase library yield.

As a starting point, we recommend performing the protocol initially with 500 ng or 2 µg of total RNA according to the expected poly(A) content. After purifying the second strand synthesis reaction (p.14), elute with 23 µl elution buffer (**EB** ○) instead of 13 µl. To determine the exact cycle number needed for your endpoint PCRs you have two options:

Option I – qPCR to determine the exact cycle number of your endpoint PCRs

Insert 10 µl (of the eluted 23 µl double stranded library, step 30) into a qPCR reaction. Simply add SYBR® Green I (or an equivalent fluorophore) to the PCR-reaction to a final concentration of 1x. For SYBR® Green I use 1 µl of a 1:500 SYBR® Green I dilution (diluted in DMSO). The total PCR reaction volume will be 21 µl. SYBR® Green I has an emission maximum at 520nm, which for some qPCR machines has to be adjusted manually. Overcycle this initial qPCR (20 cylces or even more if little input material was used) and then determine the fluorescence value at which the fluorescence reaches a plateau. Calculate where the fluorescence is at 25% from the maximum and this is the cycle number you should use for the endpoint PCR using the second half of the template. The SENSE kit is provided with enough PCR Mix and E2 to perform 2 PCR reactions for each library. There is no need to purify or analyze the overcycled PCR reaction on a Bioanalyzer. Please be aware that the post-PCR purification columns are only intended for the endpoint PCRs (8 post-PCR purification columns, plus 2 extra columns for the 8 rxn kit and 24 post-PCR purification columns + 6 extra columns for the 24 rxn kit).

Option II – endpoint PCR with one additional cycle and Bioanalyzer quantification

Insert 10 µl (of the eluted 23 µl double stranded library, step 30) into the PCR reaction and perform 9 cycles of library amplification instead of 8 (or one more cycle than listed in the table of Appendix B (p.21) depending on the column binding buffer (CBS/CBL) that was used for library purification). If the library yield is as described in Appendix B (p.21), performing the protocol on similar samples as described in the manual (with 13 µl elution buffer and 8 cycles of amplification) should generate sufficiently complex libraries. If yield is insufficient amplify the remaining 10 µl of the purified second strand synthesis reaction with 2-4 additional cycles (until an acceptable yield is reached), and increase the total RNA input accordingly in future experiments. Extra reagents for two (8 prep kit) or six (24 prep kit) additional library purifications are included.

RNA integrity

The integrity of an RNA sample can be assessed with a variety of methods. We recommend the use of a microfluidics assay such as the RNA6000 series for the 2100 Bioanalyzer (Agilent Technologies Inc.), although RNA quality can also be assessed with denaturing agarose gel electrophoresis if such a device is not available. Most microfluidics platforms will carry out an automated peak analysis and generate a quality score (RIN or RQN), and we recommend a RIN score of 8 or greater for optimal sequencing results. Typically such samples have easily detectable rRNA peaks and a comparatively low abundance of short RNAs, which can arise from both intact short transcripts as well as from RNA degradation. Libraries can also be generated from lower quality RNA, but this may lead to 3'-bias in sequencing results.

Potential contaminants

RNA samples should be free of salts, metal ions, and organic solvents which can be carried over from RNA extraction. Several sources of contamination can be detected with a UV-Vis spectrophotometer. An acceptably pure RNA sample should have an A260/A280 ratio between 1.8 and 2.1. The A260/A230 ratio should also be approximately 2. Several common contaminants including proteins, chaotropic salts, and phenol absorb strongly between 220 and 230 nm and can often be identified as peaks in this region. Contamination with any of these generates a lower A260/230 ratio. Phenol also has an absorption maximum between 250 and 280 nm which overlaps that of nucleic acid, so high 230 nm absorbance combined with a biphasic or broad peak between 250 and 280 nm may indicate contamination with phenol rather than chaotropic salts.

Genomic DNA contamination

Depending on the RNA extraction protocol used, samples may also contain significant amounts of gDNA, which is indistinguishable from RNA on a spectrophotometer. Furthermore, as many of the dyes used in RNA microfluidics assays stain single-stranded nucleic acids much more intensely than double-stranded, low to moderate amounts of gDNA may not be readily visible

with an RNA-specific microfluidics assay. We highly recommend examining all RNA samples on a denaturing agarose gel or using a fluorometric assay with DNA- and RNA-specific dyes to check samples for DNA contamination. On an agarose gel, gDNA can appear as either a dark mass which remains in the slot if relatively intact or as a high molecular weight smear if it has been sheared during extraction. SENSE libraries generated from samples containing gDNA may have an increased number of intergenic reads or lower strandedness.

The best way to avoid gDNA contamination is to use an RNA extraction protocol that does not co-isolate gDNA. However, DNA can be removed from irreplaceable samples by acidic phenol extraction or DNase I digestion. We do not recommend DNase treatment as the extended incubation with divalent cations can lead to RNA hydrolysis and decrease RNA integrity. If samples must be DNase treated, heat inactivation should be avoided and the enzyme deactivated by other means such as phenol/chloroform extraction or silica column purification.

RNA storage

If immediate RNA extraction is not possible, tissue samples can be either flash-frozen with liquid nitrogen or submerged in RNAlater® (Life Technologies Inc.) and stored at -80 °C. After extraction, RNA can be stored at -20 °C or -80 °C in 10 mM Tris pH 7.0. Avoid frequent freeze/thaw cycles as RNA might be sheared.

ERCC RNA spike-in controls

To enable the hypothesis-neutral calculation of strandedness, we highly recommend the addition of artificial transcripts of known strand orientation and concentration such as the ERCC RNA spike-in controls (Ambion Inc.). These sets of RNAs have a known strand orientation and no antisense transcripts, so the calculation of strandedness based on ERCC sequences is more accurate than calculations based on reads aligning to the genome.

8. Appendix B: Adjusting Library Size

The size of SENSE libraries can be adjusted to the desired sequencing length. This is accomplished by modulating the insert range of the library generated during RT/ligation and by using different size cut-offs during purification.

SENSE is offered with two different reverse transcription and ligation mixes to be used in step 12 of library generation. As shown in the table below **RTS** will produce libraries with shorter mean insert sizes, while **RTL** generates libraries with longer inserts. Additionally, the desired library size can be further fine-tuned by varying the ratio of short (**CBS**) to long (**CBL**) binding buffer in steps 23 and 26.

Please refer to the table below to see which column binding buffer (**CBS**, **CBL**, and mixtures thereof) is appropriate for your desired read length.

The required volumes of **CBS** and **CBL** can be added directly to the sample after second strand synthesis. The µl listed refer to the volumes needed per sample to be purified.

Depending on your selected insert range the number of PCR cycles during library amplification varies slightly (from 8 to 10 cycles). Check the table to see which cycle number is required to obtain >10 nM of library for your selected read length. All reference values shown here refer to 500 ng total RNA starting material. If using lower RNA input amounts further cycles need to be added (for RNA amount also refer to Appendix A, p.18).

Sequencing length		Ratio of CB in steps 23 + 26		Library*			Insert			library yield		Recommended PCR cycles
		CBS x	CBL y	Start [bp]	End [bp]	Mean size*	Mean size	>100 nt	> 200 nt	ng/µl	nM	
SR50	RTS	160 µl		116	700	226	110	32%	3%	1.2	8.8	8
SR100 or PE50	RTS	120 µl	40 µl	140	700	250	134	55%	6%	1.1	7.1	9
SR150	RTS	80 µl	80 µl	150	700	271	155	75%	11%	1.4	8.3	10
PE100 or SR250	RTL	120 µl	40 µl	140	1700	345	229	77%	27%	2.4	12.8	8
PE150	RTL	80 µl	80 µl	150	1700	370	254	89%	35%	1.5	7.3	8
PE250	RTL	40 µl	120 µl	160	1700	395	279	95%	43%	2.2	11.0	9
PE300	RTL		160 µl	180	1700	424	308	99%	66%	3.8	15.4	10

*For non-multiplexed libraries. Libraries prepared with internal barcodes are 10 bp longer, libraries prepared with external barcodes are 6 bp longer, and dual indexed libraries are 16 bp longer.
SR: Single-Read Sequencing PE: Paired-End Sequencing

ATTENTION: DO NOT USE **CBL** ALONE in step 23 or 26 if the library was synthesized with **RTS** as this may result in severe decrease in library yield.

9. Appendix C: Library Quality Control

Quality control of finished SENSE libraries is highly recommended and can be carried out with various methods depending on available equipment. A thorough quality control procedure should include the analysis of both the concentration and the size distribution of libraries.

Quality control methods

The analysis of a small volume of the amplified library with microcapillary electrophoresis has become the de facto standard for many NGS laboratories and generates information regarding library concentration and size distribution. Several electrophoresis platforms are available from various manufacturers. For low- to medium-throughput applications, we recommend the Agilent Bioanalyzer 2100 and High Sensitivity DNA chips (Agilent Technologies Inc.). Typically, 1 µl of SENSE library produced according to the directions in this manual can be analyzed directly on a High Sensitivity Chip. However, samples may need to be diluted to prevent detector saturation if additional PCR cycles were used.

More accurate library quantification can be achieved with custom or commercially available qPCR assays. With these assays, the relative or absolute abundance of amplifiable fragments contained in a finished SENSE library is calculated by comparing Cq values to a set of known standards. While generating more accurate quantification, these assays do not supply the user with information regarding library size distribution. The use of such an assay for quantification in combination with Bioanalyzer analysis for size distribution is highly recommended.

If microcapillary electrophoresis platforms and qPCR machines are not available, very basic quality control can also be performed by separating a small aliquot of the library on a polyacrylamide or agarose gel. Library quantification can also be performed with an inexpensive benchtop fluorometer using one of several commercially available assays. Most UV-Vis spectrophotometers are not sensitive enough at these concentrations to accurately quantify NGS libraries and should be avoided.

Typical results

SENSE kits are provided with 2 different reaction buffers **RTS** and **RTL** which generate libraries with different size ranges. Additionally, the library size can be varied depending on the column binding buffer used in steps 23 and 26 . For a detailed overview regarding library size, insert range, and yield please refer to the table in Appendix B: Adjusting Library Size (p.21).
Typical concentrations are between 7-15 nM (1.1-3.8 ng/µl), which are well suited for cluster generation without further processing. A shorter side-product caused by the direct ligation of starter/stopper heterodimers to one another is sometimes visible at ~129 bp, and should compose no more than 0-3% of the total library. Higher proportions of this side-product can indicate problems during library preparation.

A second peak in high molecular weight regions (between 1000 - 9000 bp) is an indication of overcycling. Performing the qPCR reaction to determine the cycle number of your endpoint PCR as recommended on page 18 should prevent overcycling. Still, even overcycled PCRs can be used for subsequent sequencing reactions without compromising your results. However, for further experiments using the same input RNA please adjust your cycle number accordingly or take advantage of the qPCR option.

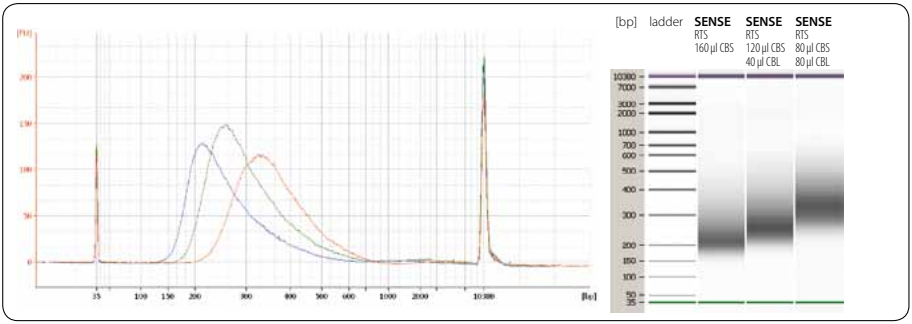


Figure 3. Bioanalyzer traces of RTS synthesized SENSE libraries purified with varying combinations of CBS and CBL. Dark blue trace: 160 µl CBS, green trace: 120 µl CBS + 40 µl CBL and red trace: 80 µl CBS + 80 µl CBL.

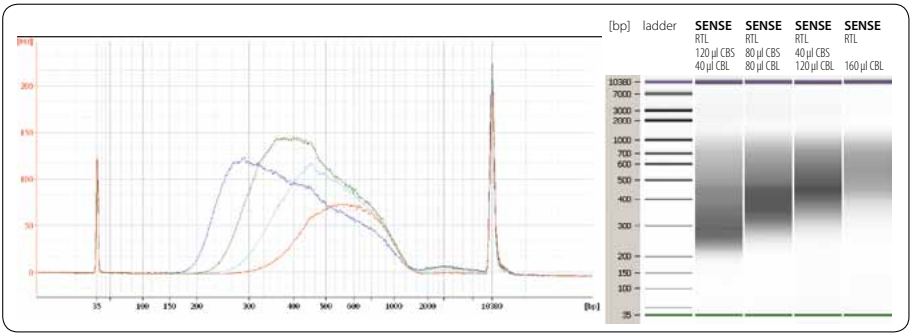


Figure 4. Bioanalyzer traces of RTL synthesized SENSE libraries purified with varying combinations of CBS and CBL. Dark blue trace: 120 µl CBS + 40 µl CBL, green trace: 80 µl CBS + 80 µl CBL, light blue trace: 40 µl CBS + 120 µl CBL, red trace: 160 µl CBL.

10. Appendix D: Multiplexing

SENSE libraries can be multiplexed. Barcodes can be introduced as standard external barcodes during the PCR amplification step or as in-line barcodes at the beginning of each read during the RT/ligation step. External barcodes require a separate sequencing reaction whereas in-line barcodes do not.

External barcodes

External barcodes can be introduced during library amplification with the SENSE External Barcode Kits (Cat. No. 003.08 or 003.24, 12 barcodes, sets A-H), allowing up to 96 samples to be sequenced per lane on an Illumina flow cell. In contrast to in-line barcodes, where the barcode is located at the beginning of both the forward (Read 1) and reverse read (Read 2), SENSE external barcodes require an additional index-specific sequencing reaction. To generate libraries with external barcodes replace the PCR mix (**PCR** ○) used during library amplification (step 32, p.15) with one of the PCR mixes (PCR01-96) supplied in the SENSE external barcode kits A-H. External barcodes are 6 nt long.

External barcode kit A (1-12)	External barcode kit B (13-24)	External barcode kit C (25-36)	External barcode kit D (37-48)	External barcode kit E (49-60)	External barcode kit F (61-72)	External barcode kit G (73-84)	External barcode kit H (85-96)
PCR01: ACATTA	PCR13: GAACCT	PCR25: ACAACG	PCR37: AGTTGA	PCR49: AGGCAT	PCR61: GAAGTG	PCR73: AACAAG	PCR85: TTGGTA
PCR02: GGTGAG	PCR14: CGGTTA	PCR26: GCGCTG	PCR38: GACGAT	PCR50: ACCTAC	PCR62: AGAATC	PCR74: AACCGA	PCR86: CAACAG
PCR03: CGAAGG	PCR15: AACGCC	PCR27: CAAGCA	PCR39: CACACT	PCR51: TGGATT	PCR63: GCGAAT	PCR75: TGGCGA	PCR87: CAATGC
PCR04: AAGACA	PCR16: CAGATG	PCR28: GTTACC	PCR40: CAGCGT	PCR52: GCAGCC	PCR64: CGATCT	PCR76: CACTAA	PCR88: GGAGGT
PCR05: TAATCG	PCR17: GATCAC	PCR29: CTCTCG	PCR41: TGCTAT	PCR53: CGCCTG	PCR65: CATCTA	PCR77: AAGCTC	PCR89: CAGGAC
PCR06: CGCAAC	PCR18: CGCGGA	PCR30: CCAATT	PCR42: TCTTAA	PCR54: CCGACC	PCR66: AAGTGG	PCR78: TACCTT	PCR90: GGCCAA
PCR07: AATAGC	PCR19: CCTAAG	PCR31: TTCGAG	PCR43: CCGCAA	PCR55: TATGTC	PCR67: TGCACG	PCR79: CTAGTC	PCR91: CTCATA
PCR08: TTAACT	PCR20: GGCTGC	PCR32: CGTCGC	PCR44: CTCCAT	PCR56: TGACAC	PCR68: TCGTTC	PCR80: AATCCG	PCR92: CCTGCT
PCR09: AATGAA	PCR21: ACCAGT	PCR33: TGTGCA	PCR45: GTCAGG	PCR57: ACAGAT	PCR69: ACACGC	PCR81: GTGTAG	PCR93: GGTATA
PCR10: GATTGT	PCR22: GTGCCA	PCR34: ACCGTG	PCR46: ACGTCT	PCR58: AGACCA	PCR70: GTAGAA	PCR82: ACTCTT	PCR94: TTCCGC
PCR11: ATAAGA	PCR23: AGATAG	PCR35: ATACTG	PCR47: GAGTCC	PCR59: GCTCGA	PCR71: AGTACT	PCR83: TCAGGA	PCR95: TAGGCT
PCR12: GCCACA	PCR24: TCGAGG	PCR36: ATGAAC	PCR48: GACATC	PCR60: ATGGCG	PCR72: GCATGG	PCR84: ATTGGT	PCR96: ATATCC

In general we recommend using a complete set of 12 barcodes for multiplexing (e.g. PCR01-12 or PCR13-24, and so on). However, if fewer barcodes are required also subsets of each set can be chosen.

When choosing subsets of barcodes it is important to make sure that both color channels used by Illumina platforms (red laser: A/C and green laser: G/T) register a signal at each nucleotide position. Listed below are some examples for subsets of barcodes.

Two samples per lane: Replace the standard 8 µl PCR mix with 4 µl PCR01 and 4 µl PCR02 for one sample and 4 µl PCR03 and 4 µl PCR04 for the second. Here two barcodes are applied to each sample in order to balance the red and green laser signals.

Four samples per lane: Replace the standard PCR mix with PCR01 for one sample, PCR02 for the second, PCR03 for the third, and PCR04 for the fourth. Apply only one PCR mix to each sample.

Eight samples per lane: Replace the standard PCR mix with PCR01 through PCR08. Apply only one PCR mix to each sample.

Twelve samples per lane: Replace the standard PCR mix with PCR01 through PCR12. Apply only one PCR mix to each sample.

Barcodes can also be combined across sets. For example the first barcode of sets A, B, C, D, E, F, G, and H can be combined in a lane mix (i.e.: PCR01/PCR13/PCR25/PCR37/PCR49/PCR61/PCR73, and PCR85), the second barcode of each set (i.e.: PCR02/PCR14/PCR26/PCR38/PCR50/PCR62/PCR74, and PCR86) can also be combined and so on.

If multiplexing fewer than 12 samples per lane it is also possible to assign a specific set of barcodes to each lane mix, in which case sequencing results can be unequivocally associated with their corresponding biological samples regardless of miscommunications or mix-ups between lanes while sequencing. Various multiplexing options are available depending on your experimental design, but care should be taken to always use sets of barcodes which give a signal in both lasers for each nucleotide position (at least one of the bases (A or C) in the red channel **AND** one of the bases (G or T) in the green channel). Furthermore, the individual libraries within a lane should be mixed in an equimolar ratio to ensure this balance.

In-line barcodes

In-line barcodes can be introduced during library preparation with the SENSE In-line Barcode Kit (Cat. No. 002.08 or 002.24), allowing up to 12 samples to be sequenced per lane on an Illumina flow cell. Indexing is performed by replacing the starter/stopper heterodimer (**ST** ●) used during reverse transcription and ligation (step 13 , p.13) with starter/stopper mixes supplied with the barcode kit (**ST1** through **ST12** ●).

Barcodes are 5 nt long and compose the first nucleotides of the read. Due to the starter/stopper heterodimer design, both the forward and reverse reads of a paired-end sequencing run will contain the barcode sequence. As indices occur just before the insert a third read is not required, so multiplexed libraries can be sequenced with standard single-end or paired-end reagents.

Illumina sequencers rely on the initial rounds of sequencing for cluster calling, and it is important that an even nucleotide balance (25 % each of A, C, G, and T) is maintained at these positions. The indices included in the SENSE multiplex barcode kit consist of three balanced sets of four barcodes, each of which (when mixed in equimolar ratios) provide sufficient diversity at each position. Only pool libraries made with complete balanced sets of barcodes (**ST1** through **ST4**, **ST5** through **ST8**, or **ST9** through **ST12**). The use of incomplete sets (for example **ST1**, **ST2**, **ST5**, and **ST9**) will result in deficiencies of some nucleotides and poor cluster calling. Some examples of potential barcoding strategies are listed below.

Two samples per lane: Replace the standard **ST** with 1 µl **ST5** and 1 µl **ST6** for one sample and **ST7** and **ST8** for the second.

Four samples per lane: Replace the standard **ST** with **ST9** for one sample, **ST10** for the second, **ST11** for the third, and **ST12** for the fourth. Any complete set of four barcodes could be used. Apply only one **ST** to each sample.

Eight samples per lane: Replace the standard **ST** with **ST5** through **ST12** (or any two complete sets of four barcodes). Apply only one ST to each sample.

Twelve samples per lane: Replace the standard **ST** with **ST1** through **ST12**. Apply only one **ST** to each sample.

When multiplexing less than 12 samples per lane it is also possible to assign a specific set of barcodes to each lane mix, in which case sequencing results can be unequivocally associated with their corresponding biological samples regardless of miscommunications or mix-ups between lanes while sequencing. Various multiplexing options are available depending on your experimental design, but care should be taken to always use complete sets of four barcodes and that the individual libraries within a set of four barcodes are mixed in an equimolar ratio.

Starter/Stopper Heterodimer	Barcode
ST*	None
ST1	CTACG
ST2	AACGT
ST3	GCTTC
ST4	TGGAA
ST5	TTCAG
ST6	GAGGA
ST7	ACTCT
ST8	CGATC
ST9	TGCGC
ST10	GATCG
ST11	ATAAT
ST12	CCGTA

*included in basic SENSE kit

Dual barcodes

External barcodes can also be combined with internal barcodes to allow up to 1152 samples to be multiplexed. To prepare dual-indexed libraries, substitute the starter/stopper heterodimer mix (**ST** ●) delivered with the standard kit for those in the SENSE internal barcoding kit (**ST1-12** ●) as described in the above section for internal barcoding. The internal barcode will be located at the beginning of the forward and reverse reads. To add the external barcode, substitute the library amplification mix (**PCR** ○) delivered with the standard kit for those in the SENSE external barcoding kits (PCR01-96) as described in the section for external barcoding. External barcodes require a third index-specific sequencing read.

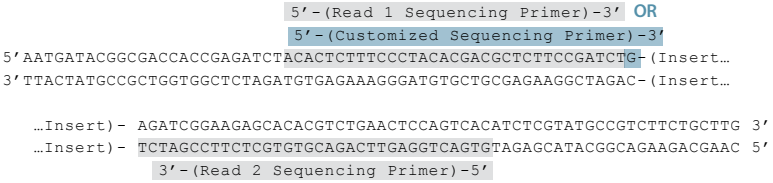
11. Appendix E: Sequencing*

General

The amount of library loaded onto the flowcell will greatly influence the number of clusters generated. Each sequencing facility has slightly different preferences of how much to load. From our experience a good starting point is to load between 7 and 14 pM of a SENSE library onto the flowcell. All SENSE libraries can be sequenced using the standard Illumina Multiplexing Read 1 Sequencing Primer. For experienced users we also offer the option to use a Customized Sequencing Primer (**CSP**) for libraries without barcoding or libraries with external barcodes, which will yield approximately 10 % more reads. The CSP must not be used with libraries with inline barcodes or dual (in-line and externally) barcoded libraries. **CSP** is included in the SENSE kit as a 100 µM stock and must be used **in the sequencing reaction at a final concentration of 0.5 µM**. As **CSP** and the standard Illumina Read 1 Sequencing Primer differ in one base (**CSP** has an additional G at the 3'end) **PhiX cannot be spiked-in if CSP is used**.
Depending on the type of barcoding used, four types of libraries can be generated: Libraries with no barcode, with external barcodes, with in-line barcodes, and with dual barcodes.

Libraries without barcodes

Here the standard starter/stopper heterodimer mix (**ST** ●) and the standard PCR mix (**PCR** ○) supplied with the basic kit (Cat. No. 001.08, 001.24) is used.



For Read 1 it is recommended to use Multiplexing Read 1 Sequencing Primer.
Optionally the Customized Sequencing Primer can be used. **ATTENTION:** Be aware that **PhiX spike-in is not possible when using CSP**. Do not use a mixture of primers such as Multiplexing Read 1 Sequencing Primer and Customized Sequencing Primer.

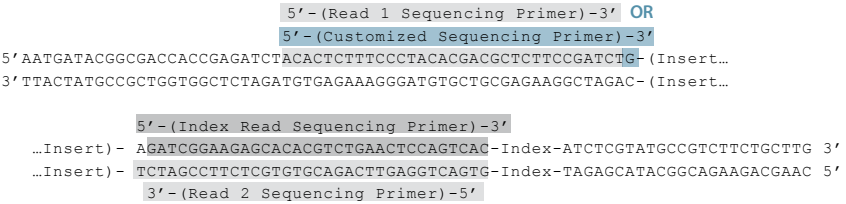
Read 1: Multiplexing Read 1 Sequencing Primer (not supplied):
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

OPTIONAL
Read 1: Customized Sequencing Primer (supplied as **CSP**, 100 µM):
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT**G** 3'

Read 2: Multiplexing Read 2 Sequencing Primer (not supplied):
5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

Libraries with external barcodes

External barcodes (6 nt) are introduced during PCR (step 32). The standard PCR mix (PCR O) supplied with the basic kit is replaced by PCR mixes (PCR01-96) from the external barcode kit (Cat. No. 003.08, 003.24, sets A-H).



For Read 1 it is recommended to use Multiplexing Read 1 Sequencing Primer.
Optionally the Customized sequencing primer can be used. **ATTENTION:** Be aware that **PhiX spike-in is not possible when using CSP**. Do not use a mixture of primers such as Multiplexing Read 1 Sequencing Primer and Customized Sequencing Primer.

Read 1: Multiplexing Read 1 Sequencing Primer (not supplied):
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

OPTIONAL
Read 1: Customized Sequencing Primer (supplied as **CSP**, 100 µM):
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT**G** 3'

Read 2: Multiplexing Read 2 Sequencing Primer (not supplied):
5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

*Note: Some nucleotide sequences shown in Appendix E may be copyrighted by Illumina, Inc.
Oligonucleotide sequences © 2007-2012 Illumina, Inc.

Libraries with in-line barcodes

In-line barcodes are 5 nt long, present on both sides of the insert and compose the first nucleotides of Read 1 and Read 2. These barcodes are introduced during reverse transcription and ligation (step 12). The standard starter/stopper heterodimer mix (ST ●) supplied with the basic kit is replaced by the starter/stopper heterodimer mixes (ST ●) supplied with the in-line barcode kit (Cat. No. 002.08 A, 002.24 A). No separate read-out of the index is required.

```
                                5'-(Read 1 Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-(Index-Insert…
3'TTACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Index-Insert…
   …Insert-Index)- AGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCTCGTATGCCGTCTTCTGCTTG 3'
   …Insert-Index)- TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTGTAGAGCATACGGCAGAAGACGAAC 5'
                    3'-(Read 2 Sequencing Primer)-5'
```

Read 1: Multiplexing Read 1 Sequencing Primer (not supplied):
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

Read 2: Multiplexing Read 2 Sequencing Primer (not supplied):
5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

Libraries with dual barcodes

For dual barcoding both external barcodes (Index 1) and in-line barcodes (Index 2) are combined. This way up to 1152 libraries can be multiplexed.

```
                                5'-(Read 1 Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-(Index 2-Insert…
3'TTACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Index 2-Insert…

                    5'-(Index Read Sequencing Primer)-3'
…Insert-Index 2)- AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-Index 1-ATCTCGTATGCCGTCTTCTGCTTG 3'
…Insert-Index 2)- TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-Index 1-TAGAGCATACGGCAGAAGACGAAC 5'
                    3'-(Read 2 Sequencing Primer)-5'
```

Read 1: Multiplexing Read 1 Sequencing Primer (not supplied):
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

Index Read: Multiplexing Index Read Sequencing Primer (not supplied):
5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3'

Read 2: Multiplexing Read 2 Sequencing Primer (not supplied):
5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

12. Appendix F: Data Analysis

This section describes a basic bioinformatics workflow for the analysis of SENSE NGS data and is kept as general as possible for integration with your standard pipeline. In contrast to most other library preparation protocols, SENSE libraries generate reads in a strand orientation opposite to the genomic reference. Reads must be re-oriented during data processing, either by conversion into their reverse complement before mapping or by inverting the directionality flag in the alignment files after mapping.

Processing raw reads

We recommend the use of a general fastq quality control tool such as FastQC or NGS QC Toolkit to examine the quality of the sequencing run. These tools can also identify over-represented sequences, which may optionally be removed from the dataset.

In order to reduce the bias introduced by the RT and hence to achieve better cluster identification on Illumina platforms, SENSE starters are not entirely random. The heptamer starter is GNNNHNG. When using Multiplex Read 1 Sequening primer the entire starter sequence is seen in the FASTQC reports. GNNN is used for cluster calling. However, the Customized Sequencing Primer (**CSP**) covers the first G of this sequence, and the following 4 bases (NNNH) will be used for cluster identification by the sequencer. This will result in a typical pattern in the FastQC reports with Gs being absent in position 4 of the sequencing reaction and a G at the 6th position.

De-multiplexing (optional)

SENSE in-line barcodes: Barcode splitting tools should be used to separate reads within a fastq file according to the given barcode sequence. The resulting fastq files can then be analyzed separately as described. It is advisable to process different barcodes in different folders in order to prevent the mix-up of files. De-multiplexing can be performed either before or after quality filtering of the reads, as these two processes do not affect each other.

The barcode is contained within the first 5 bases of the read, and should be removed after demultiplexing but before alignment. With a paired-end dataset, both the forward and the reverse reads contain the barcode.

SENSE external barcodes: The barcocde is contained in the Index Read and demultiplexing can be carried out by the standard Illumina pipeline.

Trimming

As SENSE is based on random priming, there may be a higher proportion of errors at the first nucleotides of the insert due to non-specific hybridization of the starter/stopper heterodimer to the RNA. These mismatches can lead to a lower percentage of mappable reads when using a stringent aligner, in which case it may be beneficial to trim these nucleotides. Trimming can be done with the same work-flow for both reads in a paired-end dataset. The first seven nucleotides need to be removed from Read 1 (starter side), while on the stopper side it is only six nucleotides (Read 2). If the Customized Sequencing Primer (**CSP**) was used only six nucleotides need to be removed from both reads (Read 1 and 2).

While trimming the first nucleotides introduced by the starter/stopper can decrease the number of reads of suitable length, the absolute number of mapping reads usually increases due to the improved read quality. Reads which are too short or have generally low quality scores should be removed from the set.

Alignment

At this point the filtered and trimmed reads can be reverse complemented and aligned with a short read aligner to the reference genome or assembled de novo. Alternatively, reads can be mapped first without conversion to the reverse complement and then the directionality flag in the alignment files can be inverted.

Transcriptome modeling

The resulting alignment files are used to model the transcriptome and assess transcript abundance. Further analyses are experiment-specific and can include differential expression, differential splicing, and promoter usage.

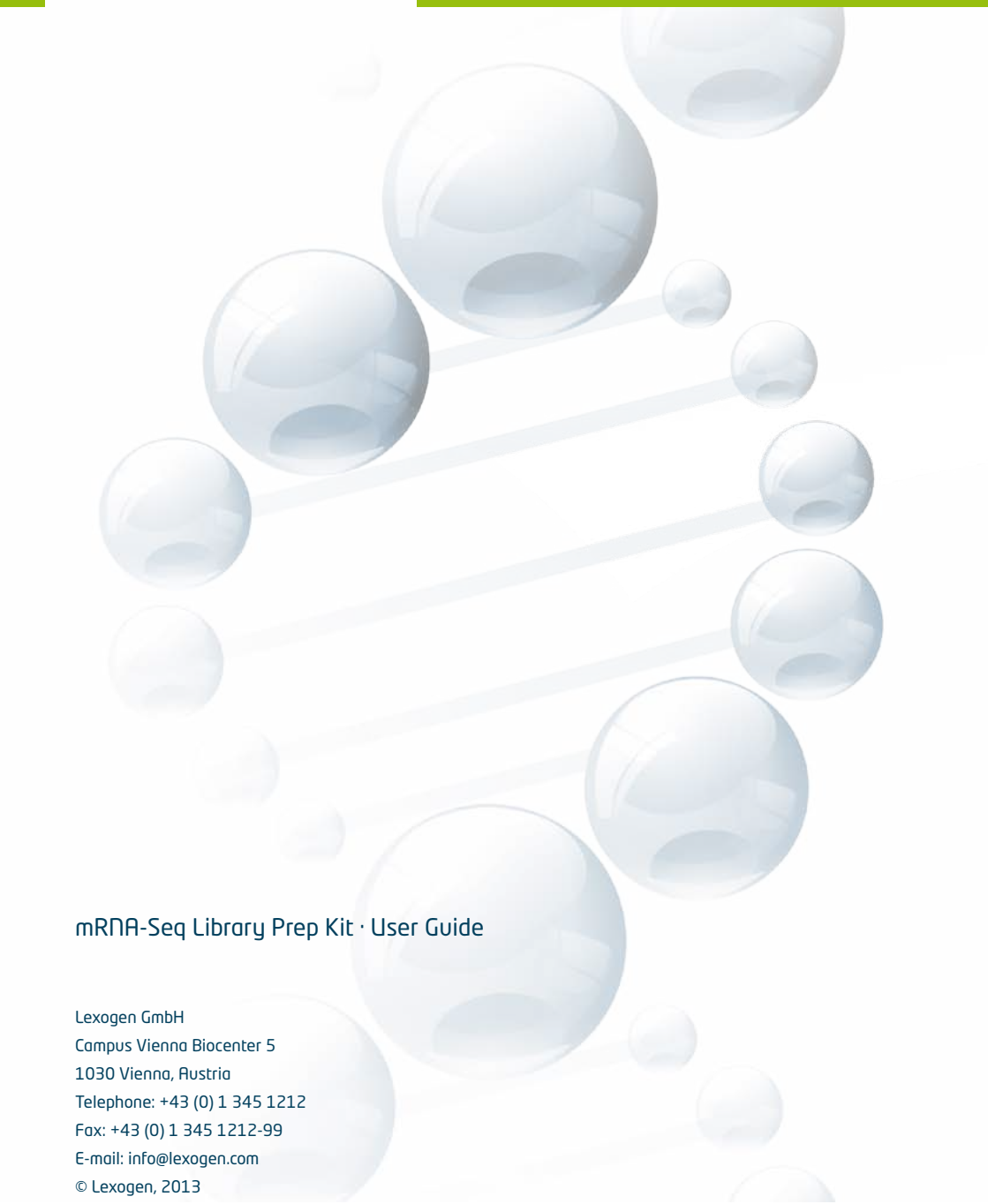
13. Appendix G: Revision History

Revision date	Change	Page
July 31st 2013	Improved beads (MS150 oligodT beads; JSR Life Sciences)	11
	Less viscous storage solution for beads, same bead amount but reduced volume and fewer pre-washes (2 now instead of 3)	11
	qPCR to determine the exact cycle number of your endpoint PCR (more E2)	18
	Fewer cycles recommended due to improved efficiency	21
	Explanation of high molecular weight peak in bioanalyzer traces	22
	Use Illumina Sequencing Primer for non-barcoded and externally barcoded SENSE libraries	28/29

14. Notes

LEXOGEN

Enabling complete transcriptome sequencing



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